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In vivo analysis of mRNA stability using the Tet-Off system in the chicken embryo

Valérie Hilgers, Olivier Pourquié, Julien Dubrulle*

Stowers Institute for Medical Research, 1000E 50th, Kansas City, MO 64110, USA

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Abstract

The rate of mRNA degradation plays an important role in the control of gene expression. The mRNA stability is mainly dependent on *cis*-regulatory elements contained in the 3' or 5' untranslated region (UTR) of the mature mRNAs, and its regulation is an efficient way to adapt the level of a given transcript in the cell. Although this process has been well studied in cell culture, little is known about mRNA stability during embryonic development. Here, we describe an assay that combines the tetracyclin-dependent inducible system Tet-Off with in ovo electroporation to monitor mRNA stability in the chick neural tube. We show, by using the GFP intensity as an indirect reporter system, that the 3'UTR of *Lunatic Fringe* strongly destabilizes transcripts, while transcripts bearing the 3'UTR of *Fgf8* are much more stable. This simple assay provides a powerful tool to study mRNA dynamics in vivo.

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Introduction

The morphogenetic events taking place during early development require rapid modulations of gene expression. The behavior of cells that build the embryo is largely dictated by interactions with their environment, and the transcriptome of embryonic cells can rapidly change depending on the signals they received. Gene regulation has been mostly studied at the transcriptional level, where genes are either activated or repressed by transcription factors that are under the influence of a variety of signals. However, gene regulation can also be regulated at the post-transcriptional level. One such regulation is the control of the stability of mRNAs (Wilusz and Wilusz, 2004). For instance, to clear up an undesired population of transcripts,

cells not only need to stop their transcription, but also need to degrade the remaining mRNAs. The expression level of a given gene is thus a direct function of its transcription rate and the degradation rate of its mRNA.

The stability of mRNAs is mainly controlled by the 3' untranslated region (UTR) of the transcript (Grzybowska et al., 2001), and the active degradation of messenger ribonucleotide acids involves a complex machinery (Tourriere et al., 2002; Wilusz et al., 2001). The first step in mRNA degradation is the shortening of the poly(A) tail by deadenylases such as the poly(A)ribonuclease (PARN) (Korner and Wahle, 1997). The poly(A) tail shortening subsequently triggers the removal of the 7-methylguanosine cap by decapping enzymes belonging to the dcp1/2 family for example (Wang and Kiledjian, 2001; Wang et al., 2002), and the mRNA is then degraded by 5' and 3' exonucleases (Bashkurov et al., 1997). Several *cis*-regulatory elements within the 3'UTR region of transcripts have been described to regulate the stability of mRNAs. One of them are the AU-rich elements (AREs), specific consensus motifs recognized by RNA binding proteins (ARE-BP) complexes (Chen and Shyu, 1995). The AREs are grouped into three classes, one

* Corresponding author. Developmental Genetics Program, Skirball Institute of Biomolecular Medicine, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA. Fax: +1 212 263 7760.

E-mail address: dubrulle@saturn.med.nyu.edu (J. Dubrulle).

class being further subdivided into five groups, based on the degree of reiteration of the pentameric motif AUUUA (Bakheet et al., 2003; Wilusz et al., 2001). The ARE-binding molecules, like AUF-1 and members of the CCCH zinc finger proteins such as Tristetraprolin (TTP) and BRF1, either promote the degradation of the mRNA (DeMaria and Brewer, 1996; Lai et al., 2000; Loflin et al., 1999; Stoecklin et al., 2002) or have a stabilizing effect, as it is the case for HuR, an ELAV-like protein (Fan and Steitz, 1998; Levy et al., 1998; Peng et al., 1998). The destabilizing activity of ARE–BP complexes is thought to be mediated in part by the recruitment of the exosome that participates in the final mRNA degradation via a 3' to 5' exonuclease and deadenylase activities (Wang and Kiledjian, 2001). It has recently been shown that the degradation of ARE-mRNAs can also be mediated by microRNAs. MiR16 is a human miRNA that displays a complementary sequence to AREs, and this miRNA cooperates with TTP in triggering mRNA degradation (Jing et al., 2005). Finally, the stabilizing or destabilizing effect of the AREs depends on the cellular context, and the mRNA stability can be regulated by several signaling pathways, such as the p38 MAPK (Winzen et al., 1999), *c-jun* N-terminal kinase (JNK) (Chen et al., 1998), MAPKAP kinase 2 (MK2) (Neininger et al., 2002), phosphatidylinositol 3-kinase (PI3-K) (Ming et al., 2001; Schmidlin et al., 2004) and the canonical Wnt pathway (Briata et al., 2003).

During vertebrate development, growing evidence suggests that the control of mRNA stability is of crucial importance in mediating embryonic processes. A good example are the molecular events taking place during somitogenesis. This process consists of generating a succession of morphologically similar mesodermal structures, the somites, along the antero-posterior axis of the embryo. The periodic formation of somites relies at least on two parameters: a so-called segmentation clock that triggers cyclic bursts of gene expression, such as *Lunatic Fringe* (*Lfng*) in chick and mouse, and generates a temporal periodic signal, and on dynamic gradients of morphogens, namely retinoic acid, FGF8 and WNT3a, that restrict the response to the segmentation clock to discrete locations within the presomitic mesoderm (Dubrulle and Pourquie, 2004a). In order to generate a cyclic expression pattern, the transcription of the so-called « cyclic genes » is periodically turned on and off (Bessho et al., 2001; Cole et al., 2002; Morales et al., 2002). In addition, the turnover rate of their mRNAs is extremely fast, the « ON » period of *Lunatic Fringe* being as short as 15 min in the chicken embryo (Dale et al., 2003). On the other hand, the gradient of FGF8 results from the establishment of a *Fgf8* mRNA gradient, which is generated via a decay mechanism from a pool of transcripts produced by progenitor cells of the presomitic mesoderm and inherited by their descendants. In this context, it has been shown that the *Fgf8* transcripts are rather stable (Dubrulle and Pourquie, 2004b).

Here, we describe an *in vivo* assay aimed at studying the stability of mRNAs in the chick embryo. This assay combines the tetracyclin-dependent Tet-Off system and *in ovo* electroporation. We first show, by performing *cFgf8* and *cLfng* 3'UTRs swapping experiments downstream of the *cFgf8* coding sequence, that the stability of *cFgf8* mRNA is mainly defined by its 3'UTR and that the 3'UTR of *LFng* has a strong destabilizing effect. To further study the properties of these 3'UTRs, we followed the kinetics of the decay of the fluorescence of an unstable GFP whose mRNA contains one of these 3'UTRs. Our experiments indicate that the GFP signal decay is at least twice faster with the *LFng* 3'UTR than with the *cFgf8* one. We propose that the use of an indirect fluorescent protein reporter is suitable to assess mRNA stability *in vivo* and to study the role of the 3'UTR in this process during embryonic development.

Material and methods

Embryos

Fertilized Hens' eggs were purchased from commercial sources, incubated at 38°C in a humidified incubator and staged according to somite numbers or Hamburger and Hamilton's (1992) tables.

Plasmids and constructs

The pTet-Off plasmid and the pTRE response vector (Clontech) have been described (Gossen and Bujard, 1992). The *Fgf8*-Flag and the unstable GFP (d1EGFP, Clontech) coding sequences have been cloned into the *EcoRI* site of pTRE (pTRE-FF and pTRE-d1EGFP respectively). The 3'UTR sequence of *cFgf8* has been cloned into the *BamHI* site of both pTRE-FF and pTRE-d1EGFP (pTRE-FF-3'*Fgf8* and pTRE-d1EGFP-3'*Fgf8*). The 3'UTR sequence of *cLfng* has been cloned into the *XbaI* site of pTRE-FF and pTRE-d1EGFP (pTRE-FF-3'*LFng* and pTRE-d1EGFP-3'*LFng*). The *cFgf8*-Flag insert has been produced by PCR on the pMIW-*cFgf8b* vector (Sato et al., 2001) using the following primers: Forward 5' AGAATTCATGGACCCCTGCTCCTC 3' and reverse 5' TCGAATTCATTGTGCATCATCGTCCTGTAGTCG 3', where the last 5 Cter amino acids of FGF8 are replaced by the Flag tag. The 3'UTR *Fgf8* and *LFng* fragments have been amplified by PCR using the following primers:

3'UTR *Fgf8* Forward 5' AAGGATCCGTGCCCCCTCGGTGGACTGACG 3' and Reverse 5' GCGGATCC-TTTGTAAAAACAGAACAAAC 3' (corresponding to nucleotides 4 to 143 out of the 186 nucleotides of the 3'UTR sequence (gi/1134969)).
3'UTR *LFng* Forward 5' GCTCTAGATCGTTGCTGTGGTATTGC 3' and Reverse 5' CGTCTAGAGCTG-

CTTTATTGGTGACG 3' (corresponding to nucleotides 102 to 1560 out of the 1583 nucleotides of the 3'UTR sequence (gi/45384413)).

Electroporation

Neural tube electroporation has been performed according to standard techniques (Itasaki et al., 1999; Momose et al., 1999) using a CUY21 square electroporator. A mixture of pTet-Off and pTRE plasmids (1 µg/µl each) was introduced in the most caudal part of the neural tube of embryos ranging from 12 to 18 somites. Four pulses (16 V, 50 ms, 100 ms intervals) were then applied, and the embryos were resealed and reincubated.

New culture and doxycycline treatment

After overnight incubation, a solution of PBS/5% Indian ink containing 9 µg/ml of doxycycline was injected between the embryo and the yolk (100 to 200 µl). Embryos were then reincubated and harvested at different times for in situ hybridization. For GFP detection, embryos as well as the intact extraembryonic tissues were dissected out in PBS and transplanted onto an albumin/agarose plate (50% thin albumen, 0.3% agarose, 66 mM NaCl, 0.15% sucrose) supplemented with 9 µg/ml of doxycycline, dorsal side up. The extraembryonic tissue was spread as much as possible on the plate, extra liquid was removed and embryos were reincubated in a humidified chamber.

In situ hybridization

In situ hybridization was carried out as previously described (Henrique et al., 1995). The *cFgf8-Flag* mRNAs containing either 3'UTRs were detected using a *Fgf8* probe (Crossley et al., 1996) and the *gfp* mRNAs using a *gfp* probe.

GFP intensity quantification

Pictures of GFP positive embryos were acquired with a Leica MZ FLIII stereomicroscope equipped with a ProgRes C14 CDD camera. Exposure time, gain and magnification were left constant for each embryo. TIFF images were imported into Photoshop, gray-scaled and trimmed to maximize the GFP over non-GFP surface ratio (image size was maintained constant for each picture of a given embryo). Images were then imported into Scion Image software, the background was subtracted, and the integrated gray intensity was calculated. For each time point, the ratio I_t/I_0 was determined and was plotted overtime in excel. The best fitting trendline was then defined, which the half-decay value was deduced from. The half-decay value is defined in this assay as the time required to lose 50% of the GFP intensity after doxycycline treatment.

Results

Control of mRNA stability is an important yet understudied aspect of the regulation of gene expression in embryonic development. In a recent study, we used actinomycin D treatment to block transcription in embryo explants to evaluate the stability of *Fgf8* and *lunatic fringe* transcripts in the chick presomitic mesoderm (Dubrulle and Pourquie, 2004b). These experiments demonstrated that *Fgf8* transcripts have a much longer persistency than *lunatic fringe* ones in this embryonic tissue. Here, we present a novel strategy to evaluate mRNA stability and particularly the role of the 3'UTR in this process in an in vivo, embryonic context, by using the tetracyclin-dependent inducible Tet-Off system coupled to electroporation in the chick neural tube. In this assay, two constructs are co-electroporated: one plasmid (pTet-Off) contains the tetracyclin-dependent transactivator (tTA) under a strong ubiquitous promoter (CMV), and the other plasmid (pTRE) includes a CMV-derived promoter which contains a tet response element conferring a tTA dependence, driving expression of the cDNA of interest. In the absence of the tetracyclin derivative doxycycline, the tTA is constitutively produced, binds to the tet operator sequences included in the tet response element and activates transcription of the downstream gene. Upon addition of doxycycline, the tTA becomes unable to bind to its promoter, therefore shutting off transcription (Gossen and Bujard, 1992). Thus, this inducible system allows to block transcription in vivo in a time-controlled manner so that it is possible to follow the decay of transcripts and proteins expressed from the pTRE plasmid over time. We chose to use the electroporation technique applied to the chick neural tube to transfect these constructs into a living embryo because it is known to be a reliable, reproducible procedure, with a high yield of recovery.

Two different constructs were introduced into the pTRE vector: both contain the coding sequence of *cFgf8* with a Flag tag located in its Cter extremity. One construct contains the endogenous 3'UTR of *cFgf8* followed by the SV40 poly(A) signal (FF 3'Fgf8), while in the other one, the 3'UTR of *cLunatic Fringe* and the SV40 poly(A) signal (FF 3'LFng) were introduced (Fig. 1A). Each of these constructs was co-electroporated with the pTet-Off plasmid in the neural tube of 2-day-old chicken embryos. The embryos were then incubated overnight to allow a steady-state level of transcription. The following morning, a solution of doxycycline was injected under the embryos that were then reincubated. Some electroporated embryos were left uninjected as controls. Embryos were then collected after different times of reincubation, ranging from 1 to 8 h and were then processed for in situ hybridization with a *cFgf8* probe (Fig. 1B).

In control embryos, a strong ectopic *cFgf8* signal could be readily detected in one side of the neural tube of embryos electroporated with either FF 3'Fgf8 ($n = 5/5$) and FF 3'LFng

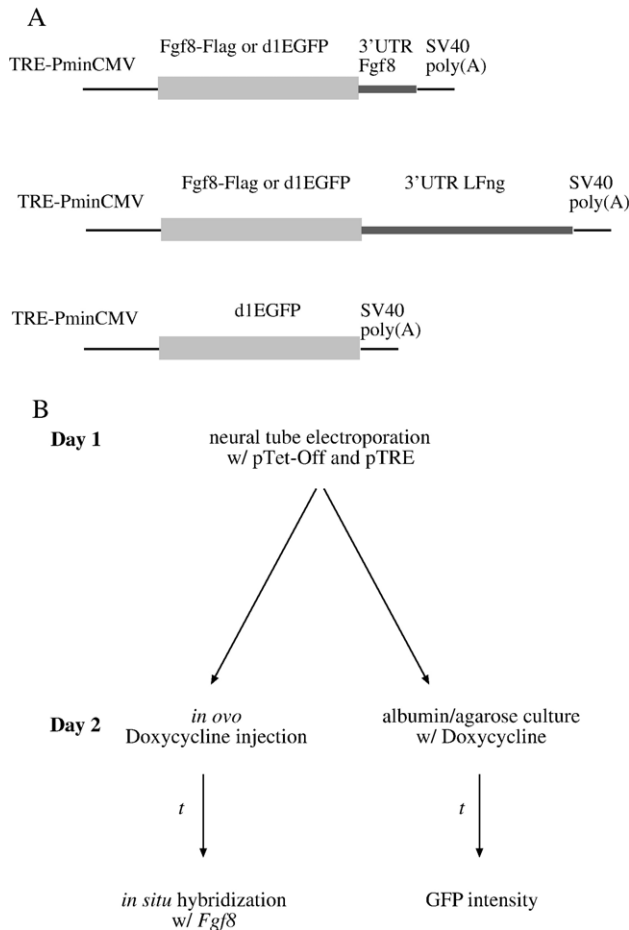


Fig. 1. Summary of the assay. (A) Scheme of the different constructs used in this study. Each contains either the *Fgf8-Flag* or the *gfp* coding sequence downstream of the promoter specifically recognized by the tetracyclin-dependent transactivator (tTA). Follows the 3'UTR of *cFgf8*, *cLFng* plus the SV40 poly(A) signal or just the SV40 poly(A) signal. (B) Design of the experiments. On day 1, neural tubes of 2-day-old chicken embryos are electroporated with the Tet-Off system. The following day, transcription of the transgene is stopped by addition of doxycycline, and transcripts contents are determined either directly by *in situ* hybridization or indirectly by analyzing the GFP intensity.

($n = 12/12$) (Figs. 2A, D), indicating that this transactivation system is able to drive expression of the transgene in the chick neural tube. In doxycycline-injected embryos, the expression of *cFgf8* was very different in FF 3'LFng compared to FF 3'Fgf8 electroporated embryos. In doxycycline-treated FF 3'LFng embryos, a majority of specimens displayed no *cFgf8* positive cells as soon as 1 to 3 h after the injection ($n = 8/11$) (data not shown and Figs. 2B, C), suggesting that the transcripts have been rapidly degraded. Furthermore, these observations suggest that doxycycline treatment fully blocks transcription from the pTRE vector, which showed no signs of leakiness in these experiments. By contrast, positive cells could be scored in FF 3'Fgf8 embryos up until 6 h after treatment ($n = 4/10$; $n = 0/8$ for FF3'LFng), suggesting that the degradation of the transcripts was slower in the FF 3'Fgf8 embryos (Figs. 2E, F). Because the only

difference between these two constructs relies on the 3'UTR, these data confirm that the 3'UTRs play a crucial role in controlling the mRNA stability. They further show that the 3'UTR of *LFng* confers instability to the *cFgf8* coding sequence, which normally displays a higher stability in the presence of its endogenous 3'UTR.

This first approach shows that such an *in vivo* inducible assay is suitable to study mRNA stability. However, the *in situ* hybridization technique is not appropriate to measure the mRNA quantity in a given tissue, and, moreover, this assay requires to compare batches of electroporated embryos fixed at different times after the doxycycline treatment. Since the efficiency of the electroporation might vary from embryo to embryo, the starting amount of the transgene mRNA varies too, and it is therefore difficult in this kind of assay to determine quantitative data such as the half-life of a given mRNA.



Fig. 2. Different mRNA stabilities of *Fgf8* transcripts bearing the 3'UTR of *LFng* or *Fgf8*. (A–F) *In situ* hybridization with a *cFgf8* probe of embryos electroporated with a pTRE-Fgf8Flag-3'UTR LFng (A–C) or pTRE-Fgf8Flag-3'UTR Fgf8 (D–F) constructs and treated for 3 to 6 h with doxycycline (B, C, E, F). (A, D) Control untreated electroporated embryos. Arrowheads in panel (F) highlight *Fgf8* positive cells in the neural tube. Dorsal view, anterior to the top.

To circumvent this issue, we decided to analyze the role of 3'UTRs in the control of mRNA stability indirectly, by using an unstable GFP as a reporter, a strategy that has been successfully used in cell culture experiments (Benjamin et al., 2004; Kakoki et al., 2004; Voon et al., 2005). We decided to use a short half-life GFP engineered by Clontech, d1EGFP. This enhanced GFP protein is fused to amino acid residues 422–461 of the mouse ornithine decarboxylase (MODC) protein (CLONTECHniques, April 1998). This C-terminal region of MODC contains a PEST amino acid sequence that targets the protein for degradation and results in rapid protein turnover. Transfection experiments of CHO-K1 cells indicate that, in the absence of cycloheximide (CHX), the fluorescence intensity of cells expressing d1EGFP is roughly the same as that of cells expressing EGFP. However, when d1EGFP-expressing cells are treated with CHX, fluorescence decreases dramatically after 4 h, indicating that d1EGFP is rapidly turned over in comparison to unmodified EGFP. The half-life of d1EGFP has been estimated to be 1 h in CHX-treated cell culture experiments (CLONTECHniques, April 1998). In this second approach, the *cFgf8-Flag* coding sequence of the former constructs has been replaced by the d1EGFP. Therefore, in this strategy, the fluorescent intensity can be followed and quantified in the same embryo over time after doxycycline treatment. Assuming that the rate of GFP protein translation, as well as its rate of degradation, is constant, the fluorescence intensity will be proportional to the amount of transcripts. This method should therefore allow to estimate the evolution of the mRNA content over time in a single embryo.

We thus compared the decay of the GFP signal after electroporation of unstable GFP constructs containing either one of the three following 3'UTR sequences plus the SV40 poly(A) signal: cFgf8 (d1EGFP 3'Fgf8), cLunatic Fringe (d1EGFP 3'LFng) or none (d1EGFP SV40) (Fig. 1A). The same experimental design was applied as previously described, except that instead of injecting doxycycline in ovo, embryos were explanted and cultured on albumin/agarose plates supplemented with doxycycline or not (Fig. 1B). This procedure allows an easy handling of the embryos to monitor the GFP signal. Every 60 to 120 min, each embryo was photographed in the GFP channel. Inverted gray-scale GFP pictures of representative embryos electroporated with the different constructs in presence or in absence of doxycycline are shown in Fig. 3.

Without doxycycline, the intensity of the GFP signal of both d1EGFP 3'Fgf8 and d1EGFP SV40 embryos increases over time, suggesting that the transcripts are accumulated and more and more GFP proteins are produced (Figs. 3D, G). By contrast, the GFP signal was generally less intense in d1EGFP 3'LFng embryos, and this signal did not significantly increase over time (Fig. 3A). Assuming that the half-life of the GFP protein is not affected by the addition of the 3'UTR of *cLFng*, this observation can be explained by the fact that cells have reached an equilibrium state between

their rate of *d1egfp* transcription and degradation so that the concentration of GFP protein stays constant.

In doxycycline-cultured embryos, we observed a decrease of the GFP intensity after prolonged incubation periods with all three constructs (Figs. 3B, E, H). However, while the kinetics of the GFP decay does not seem to be very different between d1EGFP 3'Fgf8 and SV40 embryos (Figs. 3E, H), which occurs very slowly, the GFP decay in d1EGFP 3'LFng embryos was dramatically fast (Fig. 3B). We concluded from these experiments that the rate of the GFP intensity decay was directly correlated to the degradation of the mRNAs, very rapid in the case of d1EGFP 3'LFng and much slower in the case of d1EGFP 3'Fgf8 and SV40. These observations have been confirmed by checking the presence of the GFP mRNAs after in situ hybridization at the end of the culture period. No *gfp* positive cells could be detected with any constructs after prolonged periods of treatment, showing that the doxycycline was efficient (data not shown).

In order to precisely compare the effect of the different 3'UTRs on the kinetics of the GFP signal, we determined the time required to lose half of the fluorescent signal in doxycycline-treated embryos, a value we called fluorescence half-decay, as opposed to mRNA half-life. This value therefore reflects both the degradation rate of the mRNA and the degradation rate of the reporter protein. To do so, the integrated GFP intensity was determined on a gray-scale for each embryo at the different recorded time points. This intensity was then expressed as a fraction of the intensity at t_0 and plotted over time.

Without doxycycline, the GFP signal had a strong tendency to increase in 3'Fgf8 ($n = 16$) and SV40 ($n = 10$) embryos (Figs. 3F, I), suggesting that the GFP mRNAs accumulate over time. By contrast, the variation of the GFP intensity in 3'LFng embryos ($n = 13$) was unevenly distributed, where it was either slightly increasing or decreasing (Fig. 3C). These results indicate that, in these experimental conditions, the balance between GFP translation and degradation is close to zero, suggesting that a rapid turnover of the mRNA containing the 3'UTR of *Lfn*g occurs.

In doxycycline-treated embryos, the global kinetics of GFP decay was similar between the 3'Fgf8 ($n = 9$) and SV40 ($n = 12$) embryos, with a first phase of increasing intensity that lasts approximately 3 to 4 h followed by a second phase where the intensity decreases in a regular way (Figs. 3F, I). The first phase presumably corresponds to the time required for a significant amount of *gfp* transcripts being destroyed to reach an equilibrium state between GFP protein production and degradation, while the second phase reflects the progressive decay of the mRNA, hence, the GFP signal. In 3'LFng embryos ($n = 15$), we could not detect the first phase, and the GFP intensity almost directly begins to decay after culture on doxycycline plates (Fig. 3C). Based on the best fitting trendlines for each experimental conditions (all polynomials, $R^2 = 0.83, 0.55$

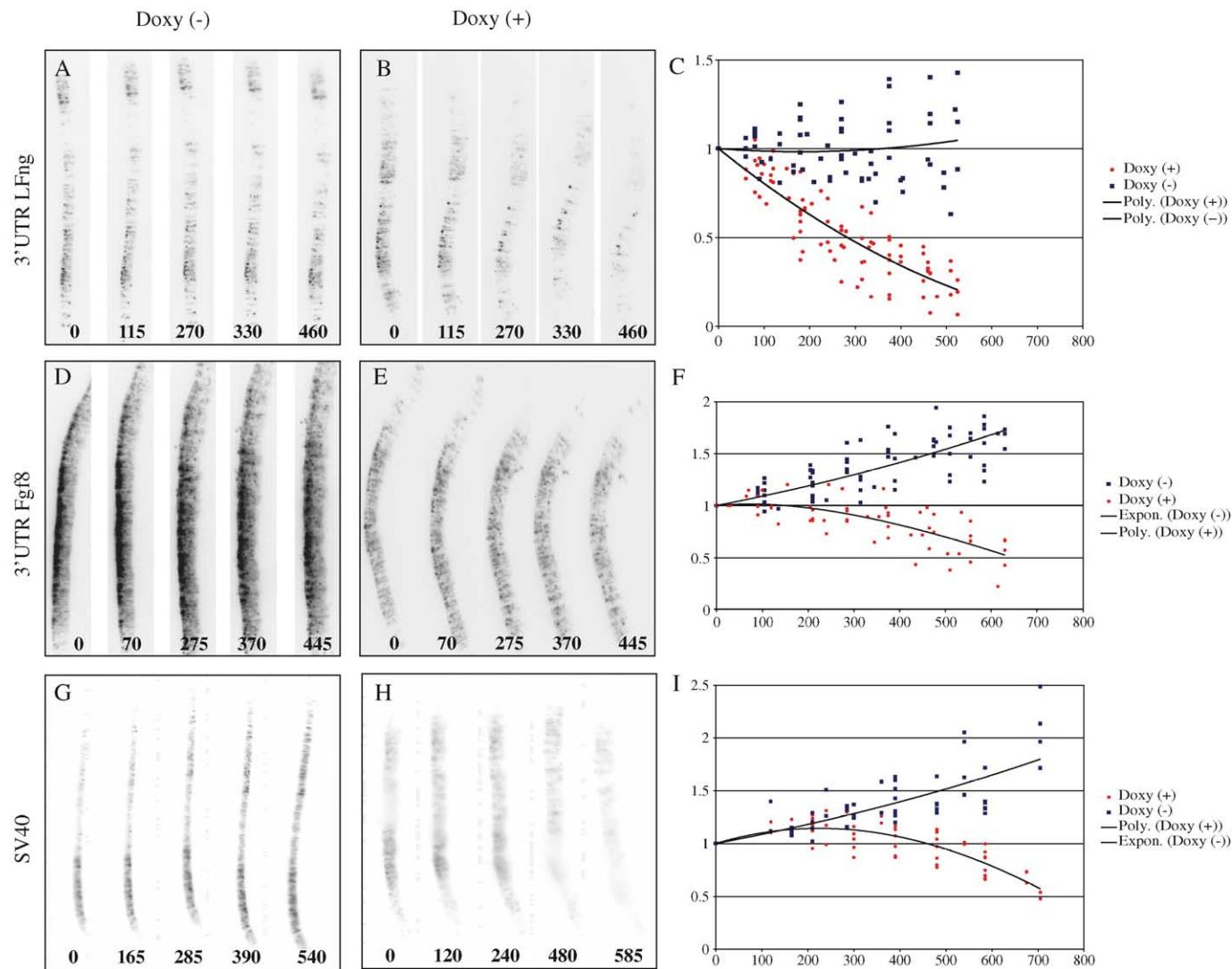


Fig. 3. Time course analysis of the kinetics of the GFP intensity decay. (A, B, D, E, G, H) Examples of neural tubes electroporated with pTRE-GFP-3'UTR *Lfng* (A, B), pTRE-GFP-3'UTR *Fgf8* (D, E) or pTRE-GFP-3'UTR SV40 (G, H) treated with doxycycline (B, E, H) or untreated (A, D, G). Each panel shows a succession of pictures (inverted gray-scaled images of the GFP signal) of a same embryo recorded at different time points (in minutes, indicated at the bottom of each image). Dorsal view, anterior to the top. (C, F, I). Variation of the relative GFP intensity (I_t/I_0) over time (in min) for pTRE-GFP-3'UTR *Lfng* (C), pTRE-GFP-3'UTR *Fgf8* (F) and pTRE-GFP-3'UTR SV40 (I) with (red squares) or without (blue dots) doxycycline treatment. Poly., polynomial trendline, Expo., exponential trendline.

and 0.66 for 3'LFng, 3'Fgf8 and SV40, respectively), we were able to determine the fluorescence half-decay value, which is 750 min for SV40, 612 min for 3'Fgf8 and 272 min for 3'LFng.

All these results show that the 3'UTR sequence of *LFng* has a strong destabilizing effect on two different coding mRNAs (*Fgf8*-Flag and GFP), while the *Fgf8* one has a stabilizing effect, at least in the same range as mRNAs that only bear the SV40 poly(A) signal.

Discussion

In this report, we describe an assay to study the turnover rate of mRNAs in the chicken embryo. As a proof of principle, we compared the effect of the 3'UTR of *cLFng* and *cFgf8* on the decay of an unstable GFP reporter. We showed that addition of the 3'UTR of *LFng* triggers a rapid degradation of the GFP mRNA, as assessed indirectly by the kinetics of the GFP intensity decay after its transcription was blocked by using the doxycycline inducible Tet-Off system.

AU-rich elements that promote or inhibit mRNA degradation have been identified (Gingerich et al., 2004), and it is possible that these elements are responsible for the instability of transcripts containing the 3'UTR region of *cLFng*. In the chick, mouse and human *Lunatic Fringe* gene, this region is more than 1.0 kb long and possesses in all of these species, 1 or 2 AREs ((Johnston et al., 1997; Laufer et al., 1997), boxed in Supplementary Fig. 1). Further experiments will be required to precisely determine whether these elements are indeed responsible for conferring instability, but the striking similarity of the 3'UTR in all these species suggests that this characteristic of *LFng* transcripts might be conserved among vertebrates. This is at least true for the chick and mouse *LFng* mRNAs in the presomitic mesoderm, which are both expressed in a periodic fashion (Forsberg et al., 1998; McGrew et al., 1998). However, emerging evidence suggest that the AREs can also mediate translational repression (Grosset et al., 2004; Jarzembowski et al., 1999; Mukhopadhyay et al., 2003), and we cannot totally exclude that the rate of GFP intensity decay with constructs containing the 3'UTR of *LFng* is in part due to such a translational repression process. Still, the fact that the *LFng* 3'UTR-containing mRNAs are rapidly cleared out, as assessed by in situ hybridization, suggest that the main factor contributing to the loss of GFP signal is the rapid reduction of the mRNA content. Our assay provides a simple means to dissect the function of the 3'UTR domains of *lunatic fringe* in a biologically relevant context.

By contrast, the chick, mouse and human *Fgf8* 3'UTRs are very short, less than 200 bp (Crossley et al., 1996; Tanaka et al., 1992, 1995), and whereas a surprising degree of conservation is seen between the three species, no specific features can be detected in their sequence, except

some conserved GU-rich stretches (Supplementary Fig. 1). It remains to be determined whether the observed stability conferred by these 3'UTRs is regulated by RNA binding protein complexes or whether this is a default status due to a lack of regulatory *cis*-elements. However, we cannot exclude that alternative poly(A) signals within the *Fgf8* locus that might drive the transcription of a different 3'UTR exists, which contains such elements. It is interesting to note that the 3'UTR of the β globin gene, which is known to confer high stability to mRNAs (Xu et al., 1998), is also very short, less than 200 bp, which is similar to the length of that of *fgf8* one. The insulin mRNA is also very stable (Welsh et al., 1985), and its 3'UTR is less than 100 bp in length, which nonetheless contains pyrimidine-rich motifs recognized by stabilizing proteins upon glucose stimulation (Tillmar et al., 2002).

Recently, Kakoki and collaborators have studied the effect of different 3'UTRs on the expression level of a single copy of GFP recombined in a fixed locus in ES cells (Kakoki et al., 2004). They have shown that the 3'UTR region has a strong influence on the level of detected GFP intensity. They noted a direct correlation between the levels of GFP mRNAs, their half-life and the fluorescent intensity: shorter half-life mRNAs induced by a destabilizing 3'UTR such as *c-Fos* lead to a lower mRNA level and less fluorescence (Kakoki et al., 2004). Accordingly, we also found that the GFP signal was less intense in the neural tube of d1EGFP 3'LFng electroporated embryos. Moreover, their study pointed out a strong enhancement of mRNA stability, which was doubled, when the GU-rich elements (GRE) downstream of the poly(A) site of a given 3'UTR were added to their constructs. These GREs recruit cleavage stimulation factors required for efficient polyadenylation (de Vries et al., 2000; Gil and Proudfoot, 1987; McLauchlan et al., 1985). Such a putative GRE (see Supplementary Fig. 2) can be identified a hundred base pairs downstream of the *Fgf8* poly(A) site, and it would be interesting in our assay to test whether the addition of this sequence can further extend the stability of the transcripts. It is also possible that, in our constructs, the addition of the SV40 poly(A) sequence downstream to the 3'UTR of *Fgf8* masks some alternative yet undefined mRNA processing events that naturally take place during *Fgf8* transcripts synthesis.

This assay provides a powerful tool to assess the turnover rate and stability of different mRNA in their relevant embryonic context and to analyze the factors involved in the regulation of this process. Addressing the exact mRNA half-life of genes expressed during development using cell culture is not always relevant since it is well known that the stability of a given mRNA can greatly vary depending on the cellular or developmental environment (Briata et al., 2003; Schwartz et al., 1992). Such an analysis in cell culture may therefore be undermined by the lack of regulators specific to the embryonic context. For example, it has been shown that the half-life of the neurofilaments mRNA is as high as 4 days in cultured primary DRG neurons, whereas it

is less than 24 h in PC12 cells (Schwartz et al., 1992). In the same line, Pitx2 transcripts are intrinsically labile while they are stabilized by Wnt signaling during development (Briata et al., 2003).

For a first approach, we have applied this experimental system to the chick neural tube because of the easiness of the electroporation technique in this tissue. This assay can nevertheless be applied to any tissues where electroporation has been made efficient (Dubrulle et al., 2001; Grapin-Botton et al., 2001; Scaal et al., 2004). The comparison of mRNA degradation rates in different tissues would give further important information about the tissue-specific factors and mechanisms involved in mRNA turnover. Finally, the Tet-Off system can also be used to study the turnover of a given protein. Assuming that the *LFng* 3'UTR will rapidly trigger the degradation of the mRNAs, adding this sequence downstream of any given coding sequence might allow to follow precisely the turnover rate of the translated product, which might be a useful alternative to cycloheximide treatment and its adverse effects on cell metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.05.021.

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